Ski is involved in transcriptional regulation by the repressor and full-length forms of Gli3

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Transcription factor Glioblastoma-3 (Gli3) is cleaved in the anterior region of the limb bud to generate its repressor form. In contrast, *Sonic hedgehog* (*Shh*) signaling from the posterior zone of polarizing activity blocks Gli3 processing and then induces the expression of Gli3 target genes, including *Gli1*. Here we report that the Ski corepressor binds to Gli3 and recruits the histone deacetylase complex. The Gli3-mediated repression was impaired by anti-Ski antibody and in *Ski*-deficient fibroblasts, and Shh-induced *Gli1* gene transcription mediated by full-length Gli3 was inhibited by Ski. Furthermore, a *Ski* mutation enhanced the digit abnormalities caused by the *Gli3* gene mutation. Thus, Ski plays an important role in pattern formation.

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In *Drosophila,* a transcription factor Cubitus interruptus (Ci) mediates Hedgehog (Hh) signaling (Alexandre et al. 1996; Domíguez et al. 1996). In the absence of Hh signaling, Ci is processed into a repressor, whereas Hh signaling prevents this Ci cleavage, generating a full-length Ci activator (Aza-Blanc et al. 1997). In mice, three Ci-related transcription factors (Gli1, Gli2, and Gli3) have been identified (Ruppert et al. 1990). Glioblastoma-3 (Gli3) is processed to a repressor form ($Gli3^{Rep}$) in a manner similar to Ci (Dai et al. 1999; Ruiz-I-Altaba 1999; Shin et al. 1999; Wang et al. 2000), whereas Gli1 is not (Dai et al. 1999). Overexpression of Gli1 in cultured cells or transgenic embryos can induce transcription of Hh target genes in the absence of Hh activity (Hynes et al. 1997; Sasaki et al. 1997; Ruiz-I-Altaba 1999). Sonic hedgehog (Shh) up-regulates *Gli1* transcription but down-regulates *Gli3* expression (Marigo et al. 1996; Lee et al. 1997). Molecular analysis suggests that Gli3 can be processed into a repressor form ($Gli3^{Rep}$) that suppresses the *Gli1* promoter, whereas the full-length form of Gli3 (FL-Gli3) directly mediates the activation of a *Gli1* promoter in response to a Shh signal (Dai et al. 1999). Gli3 plays an important role in the development of limb bud, and mice with a mutation in *Gli3* have dominant preaxial polydactyly (Hui and Joyner 1993).

Ski and its related protein Sno act as corepressors, and directly bind to two other corepressors, N-CoR/SMRT and mSin3A (Nomura et al. 1999). These three corepressors (N-CoR/SMRT, mSin3, and Ski/Sno) form a complex with histone deacetylases (HDACs) and are necessary for the transcriptional repression mediated by nuclear hormone receptors, Mad, and possibly other repressors. Ski also directly binds to Smad proteins, which induce the transcription of target genes on TGF-β (tumor growth factor) stimulation (Massagué and Wotton 2000.). By recruiting the HDAC complex to Smad proteins, Ski inhibits TGF-β signaling. The *Ski*-deficient mice display various abnormalities of pattern formation depending on the genetic background (Berk et al. 1997; Colmenares et al. 2002). However, the molecular mechanism of those defects remains unknown. In this study, we have demonstrated that Ski is required for the $Gli3^{Rep}$-mediated repression, and it negatively regulates the FL-Gli3-induced transcriptional activation.

Results and Discussion

Identification of Ski as a $Gli3^{Rep}$-binding protein

To identify the $Gli3^{Rep}$-interacting factor(s), we performed yeast two-hybrid screening using the N-terminal region of Gli3 or Gli2 as bait. Five *Ski* clones and three *Sno* clones were isolated, suggesting that Ski might play an important role in Gli3-mediated transcriptional regulation. To identify the Ski-interacting region in Gli3, we performed the glutatione S-transferase (GST) pull-down assay using various forms of in vitro translated Gli3 and GST–Ski fusion (Fig. 1A). The N-terminal region of Gli3 contains the repressor domain, whereas the C-terminal half contains the activation domain (Dai et al. 1999). The results indicated that the repressor domain of Gli3 (amino acids 1–397) interacts with Ski. Because a deletion of one-third of the C-terminal proximal side of the repressor domain partly decreased affinity for Ski, the repressor domain may have multiple binding sites for Ski. Similar to the case of Gli3, Ski also bound to the N-terminal repressor domain of Gli2 (Fig. 1A). To identify the Gli3-interacting domain in Ski, we used various forms of in vitro translated Ski in GST pull-down assays with a GST fusion of the repressor domain of Gli3 (Gli3CT2; Fig. 1B). The results indicated that the region between amino acids 197 and 261 of Ski mediates the interaction with Gli3CT2. This region shows a high degree of homology (63%) with Sno. Consistent with this, Sno was also capable of binding efficiently to Gli3CT2 (data not shown).

[*Keywords*: Ski corepressor; Shh signaling; Gli3; *Gli1* promoter; limb bud]
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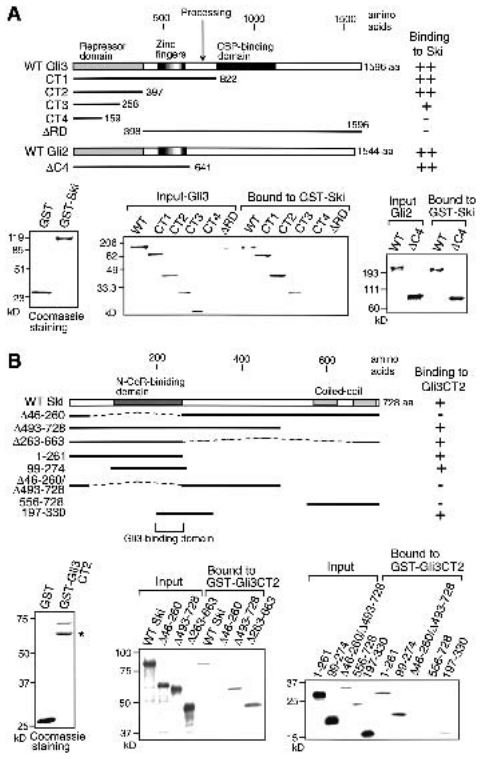

Figure 1. Binding of Ski to Gli3 and Gli2. (*A*) The repressor domains of Gli3 and Gli2 bind to Ski. The relative binding activities are designated ++, +, and –, which indicate the binding of 5%–9%, 3%, and <0.5% of the input protein, respectively. In the *lower-left* panel, the GST–Ski fusion and GST proteins that bound to the glutathione beads were analyzed by SDS-PAGE followed by Coomassie blue staining. In the *lower-right* panel, the in vitro translated Gli3 and Gli2 derivatives (input) and those that bound to GST–Ski were analyzed by SDS-PAGE followed by autoradiography. In the input lanes, the amount of each Gli3 derivative was 10% of that used for the binding assay. (*B*) Identification of the Gli3-binding domain in the Ski molecule. Binding of various forms of in vitro translated Ski to the GST–Gli3CT2 resin containing N-terminal 397 amino acids of Gli3 was examined. The relative binding activities are designated + and –, which indicate the binding of 3%–5% and <0.5% of the input protein, respectively. The band indicated by an asterisk is a GST–Gli3CT2 degradation product.

Ski binds to both Gli3^{Rep} and FL-Gli3

To investigate the interaction between Ski and Gli3 in mammalian cells, we performed coimmunoprecipitations using 293T cells (Fig. 2A). When FL-Gli3 was coexpressed with the catalytic subunit of cAMP-dependent protein kinase (PKA), FL-Gli3 was efficiently processed into GLI3^{Rep}, as reported (Dai et al. 1999). The anti-Ski antibodies coprecipitated both FL-Gli3 and Gli3^{Rep}, whereas control anti-β-galactosidase antibody did not. In similar experiments, Gli1 was not coprecipitated with Ski. In addition, a two-hybrid assay was performed in mammalian cells using the Ski–VP16 fusion, which consists of the N-terminal 492 amino acids of Ski and the VP16 transcriptional activation domain (Fig. 2B). The Gli site-containing luciferase reporter and the Ski–VP16 expression plasmid were transfected into MNS-70 cells together with the plasmids expressing either FL-Gli3 or Gli3ΔC containing the N-terminal 649 amino acids of Gli3, which has a structure similar to Gli3^{Rep}. The Ski–VP16 fusion stimulated FL-Gli3 activity 3.9-fold (relative luciferase activity: 0.74 and 2.87) and Gli3ΔC activity by 18.8-fold (relative luciferase activity: 0.19 and 3.57; see Supplementary Table 1). These results indicate that Ski interacts with the N-terminal region of Gli3.

To further confirm the Ski–Gli3 interaction, we investigated the colocalization of both proteins in CV-1 cells (Fig. 2C). When Ski was expressed alone, it was localized to a dot-like nuclear structure, as reported (Nomura et al.

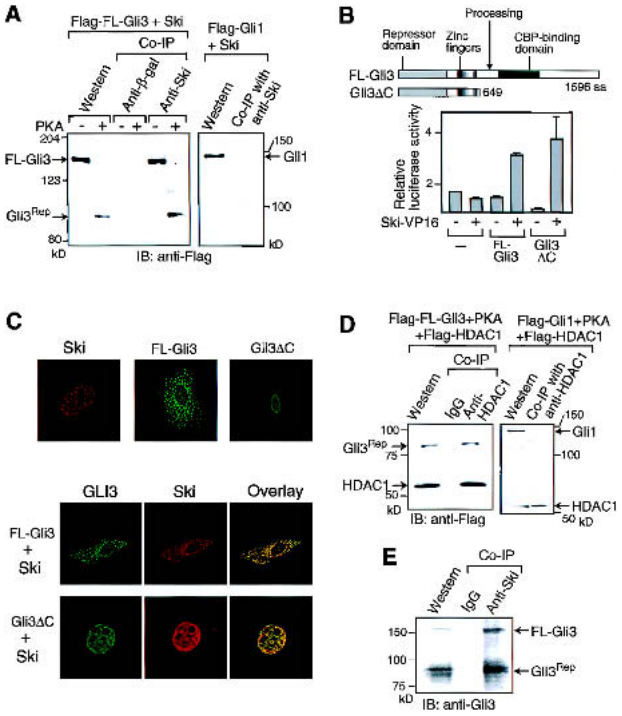

Figure 2. In vivo association between Gli3 and the Ski–HDAC1 complex. (*A*) Coimmunoprecipitation of Ski with Gli3. Lysates were prepared from 293T cells transfected with the Ski and Flag–FL-Gli3 expression plasmids together with the PKA expression plasmid (+). Lysates were precipitated by anti-Ski or anti-β-galactosidase antibodies, and the immunocomplexes were analyzed by Western blotting using anti-Flag antibodies. Samples from the lysates were also used directly for Western blotting. Similar experiments were also done using the Ski and Flag–Gli1 expression plasmids (*right*). (*B*) Two hybrid assays in mammalian cells. MNS70 cells were cotransfected with the Gli site-containing reporter, the plasmid to express FL-Gli3 or GLI3ΔC, and the Ski–VP16 expression plasmid. The average degree of activation observed in two experiments is indicated with standard deviations. (*C*) Colocalization of Gli3 and Ski. (*Top*) CV-1 cells were transfected with each of the plasmids to express Ski, Flag–FL-Gli3, or Flag–Gli3ΔC, and cells were then immunostained with the corresponding antibodies. Localizations were revealed by staining with rhodamine- or FITC-conjugated secondary antibodies. The signals were analyzed by deconvolution microscopy, and representative optical sections are shown. (*Bottom*) CV-1 cells were transfected with the Ski expression plasmid together with the plasmid to express Flag–FL-Gli3 or Flag–Gli3ΔC, and immunostaining was performed. In the right panels, signals for both proteins are superimposed. (*D*) Coimmunoprecipitation of HDAC1 with Gli3. Lysates were prepared from 293T cells transfected with a mixture of plasmids to express Flag–FL-Gli3, PKA, and Flag–HDAC1. Lysates were immunoprecipitated by anti-HDAC1 or anti-β-galactosidase antibodies, and the immunocomplexes were analyzed by Western blotting using anti-Flag antibodies. Similar experiments were also done using the Flag–Gli1 and Flag–HDAC1 expression plasmids (*right*). (*E*) Coimmunoprecipitation of endogenous Gli3 and Ski. Lysates were prepared from the 11.5 dpc mouse fetuses, and immunoprecipitated by anti-Ski or control IgG, and the immunocomplexes were analyzed by Western blotting using anti-Gli3 antibodies.

1999). FL-Gli3 was localized to dot-like structures in both cytoplasm and nucleus, whereas Gli3ΔC staining only displayed a punctate pattern in the nucleus. When Ski was coexpressed with FL-Gli3, Ski was localized to both cytoplasm and nucleus, and the signals of the two proteins overlapped. Coexpression of Ski with Gli3ΔC also led to the complete overlap of the two signals, and Ski proteins showed a broader distribution pattern in the nucleus compared with those expressed alone. Because Ski forms a complex with HDAC1 (Nomura et al. 1999), we examined whether $Gli3^{Rep}$ forms a complex with HDAC1 by coimmunoprecipitation (Fig. 2D). The plasmids to express FL-Gli3 and HDAC1 were transfected into 293T cells together with the plasmid encoding the catalytic subunit of PKA, and immunoprecipitation was performed using anti-HDAC1 antibody. $Gli3^{Rep}$ was efficiently coprecipitated with HDAC1, indicating that HDAC1 and $GLI3^{Rep}$ associate with one another. In similar experiments, Gli1 was not coprecipitated with HDAC1. To further confirm the in vivo interaction between Gli3 and Ski, we performed coimmunoprecipitation of endogenous Gli3 with Ski using lysates prepared from E11.5 mouse embryos (Fig. 2E). Anti-Ski antibodies coprecipitated both FL-Gli3 and $Gli3^{Rep}$.

Ski is required for $Gli3^{Rep}$-dependent transcriptional repression

Interaction of Ski with $Gli3^{Rep}$ suggested that Ski is required for the $Gli3^{Rep}$-dependent transcriptional repression. We investigated whether the Ski mutants abrogate $GLI3^{Rep}$-dependent repression in a dominant negative fashion using a neural stem cell line, MNS-70, that is able to express different sets of ventral-specific genes in response to Shh (Fig. 3A). In the luciferase reporter assays, the Gal4–Gli3CT2 fusion, which consists of the Gal4 DNA-binding domain fused to the N-terminal repressor domain of Gli3, strongly repressed transcription from the Gal4 site-containing reporter. Gal4–Gli3CT2-induced repression was abrogated by the C-terminal deleted form of Ski (Δ493-728) in a dose-dependent manner, neither by the wild-type Ski nor by the N-terminal deleted form (Δ46-260), which cannot bind to Gli3. Because the C-terminal deleted form of Ski binds to Gli3 but not to corepressor mSin3A, it may disrupt the Gli3-corepressors-HDAC complex. SkiΔ46–260 may not efficiently mask the surface of mSin3A molecule in vivo because the mSin3A forms a complex with many other proteins. We also performed luciferase reporter assays using mouse embryonic fibroblasts (MEFs) prepared from wild-type or *Ski*-deficient embryos (Shinagawa et al. 2001; Fig. 3B). Gal4–Gli3CT2 efficiently repressed luciferase expression from the Gal4 site-containing reporter in wild-type MEFs, but not in *Ski*-deficient MEFs. In addition, similar results were obtained by using Gal4–Gli2N containing the N-terminal 308 amino acids of Gli2. Thus, a loss of Ski abrogated the Gli3CT2- or Gli2N-induced transcriptional repression, suggesting that the amounts of Sno in MEFs are relatively low. In fact, we found that the relative levels of Sno compared with Ski are lower in MEFs than that in E12.5 embryos (Supplementary Fig. 1).

To further confirm that Ski is required for $Gli3^{Rep}$-dependent repression, antibodies were coinjected into Rat-1 cells along with a Gal4–*lacZ* reporter construct containing the TK promoter and the Gal4-binding sites, and/or the Gal4–Gli3CT2 expression plasmid (Fig. 3C).

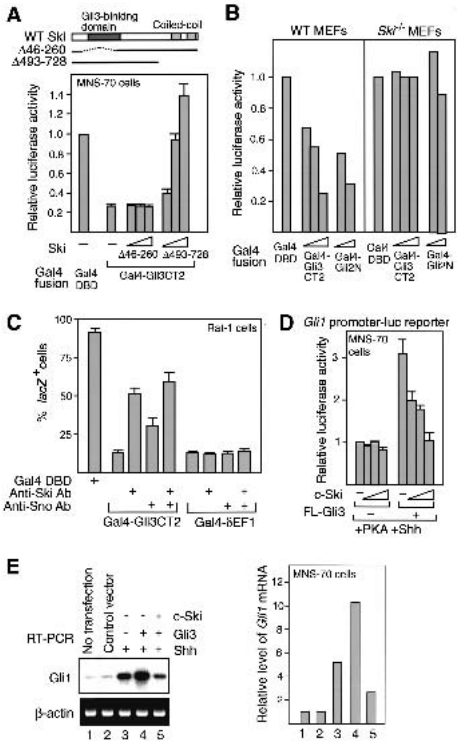

Figure 3. Role of Ski in the $Gli3^{Rep}$- and FL-Gli3-mediated transcriptional regulation. (*A*) The Ski mutant abrogates Gli3-mediated repression. A mixture containing the Gal 4 site-containing luciferase reporter, the Gal4–Gli3CT2 or Gal4 expression plasmid, and the plasmid encoding each Ski mutant was transfected into MNS-70 cells, and luciferase activity was measured. The average result of three experiments is shown. (*B*) Repressor activities of Gli3 and Gli2 in *Ski*-deficient MEFs. Wild-type or *Ski*-deficient MEFs were transfected with the Gal4 site-containing luciferase reporter and the plasmid expressing Gal4–Gli3CT2, Gal4–Gli2N, or Gal4, and luciferase activities were measured. (*C*) Abrogation of Gli3-induced repression by anti-Ski/Sno antibodies. The Gal4 site-containing *lacZ* reporter was injected with the plasmid encoding Gal4, Gal4–Gli3CT2, or Gal4–δEF1. The effect of anti-Ski/Sno antibodies on the number of *LacZ*-positive cells was examined. The bar graph represents the average results from three experiments. (*D*) Effect of Ski on the Shh- and FL-Gli3-mediated activation of *Gli1* promoter. MNS-70 cells were transfected with the *Gli1* promoter-containing luciferase reporter, the plasmids to express FL-Gli3, PKA, and Shh, and various amounts of the Ski expression plasmid, and then luciferase activities were measured. The average result from three experiments is shown. (*E*) Inhibition of Shh-induced *Gli1* expression by c-Ski. MNS-70 cells were transfected with a mixture of the Shh expression plasmid and the plasmid to express GLI3 and c-Ski. *Gli1* expression was analyzed by RT–PCR. Cytoplasmic β-actin was used as a control. On the right, the degree of *Gli1* expression is indicated by a bar graph.

Injection of the reporter alone into Rat-1 cells gave rise to many *lacZ*-positive cells. Coinjection of this *lacZ* reporter with the Gal4–Gli3CT2 expression plasmid resulted in a decrease in the number of *lacZ*-positive cells. This decrease was relieved partially by coinjection of anti-Ski or anti-Sno antibodies, and more significantly by the coinjection of both antibodies. The incomplete abrogation of Gal–Gli3CT2 function even after coinjection of both antibodies may be due to the presence of other Ski-related protein(s). Coinjection of both antibodies did not affect the decrease in the number of *lacZ*-positive cells mediated by Gal-δEF1, which was previously shown not to use Ski/Sno (Nomura et al. 1999).

Ski negatively regulates the Shh-induced activation of Gli1 promoter mediated by FL-Gli3

Ski binds not only to $Gli3^{Rep}$ but also to FL-Gli3. We examined whether Shh- and FL-Gli3-dependent activation of the *Gli1* promoter is inhibited by Ski (Fig. 3D). As reported (Dai et al. 1999), coexpression of Shh and Gli3 in MNS-70 cells transfected with the *Gli1* promoter luciferase reporter enhanced the luciferase expression. Coexpression of Ski inhibited this activation in a dose-dependent manner. Thus, Ski also inhibits Shh- and FL-Gli3-dependent activation of the *Gli1* promoter. We further investigated whether Ski inhibits the Shh-dependent endogenous *Gli1* induction mediated by Gli3 in MNS-70 cells (Fig. 3E). As reported previously (Dai et al. 1999), ectopic expression of Shh alone or together with Gli3 in transfected MNS-70 cells induces expression of the endogenous *Gli1* gene 5.2- and 10.2-fold, respectively. Coexpression of c-Ski with Shh and Gli3 significantly lowered the level of induction of *Gli1* mRNA by about 3.8-fold. These results further confirm that c-Ski negatively regulates the Shh-dependent transcriptional activation of *Gli1*.

Genetic interaction between Ski and Gli3

To test for a genetic interaction between *Ski* and *Gli3*, we analyzed the skeletons of limbs of double mutant mice (Fig. 4A; Table 1)1. *Ski* heterozygous mutant mice ($Ski^-/+$) were crossed with *Gli3* heterozygotes ($Gli3^{Xtj}/+$). In addition, $Gli3^{Xtj}/+Ski^-/+$ mice were also mated with $Gli3^{Xtj}/+;Ski^-/+$ or $Ski^-/+$ mice. As reported (Hui and Joyner 1993; Dunn et al. 1997), the $Gli3^{Xtj}/+$ mice showed mainly one extra digit (94%–95%) and rarely two (1%–2%). Although $Ski^-/+$ mice showed no limb defects, the limb of $Gli3^{Xtj}/+;Ski^-/+$ double heterozygous mice had one or two extra digits, and the frequency of two extra digits (75% of forelimb and 11% of hindlimb, Table 1) was higher than that of $Gli3^{Xtj}/+$ mice. Furthermore, a small posterior outgrowth was observed in 58% of the forelimb of $Gli3^{Xtj}/+;Ski^-/+$ mice, but not in the forelimb of $Gli3^{Xtj}/+$ mice (Fig. 4A; Table 1). The posterior outgrowth was found in 93% of the forelimb of Ski^-/Ski^- mice, as reported recently (Colmenares et al. 2002) and also in the *Gli1/Gli2* homozygous mutant (Park et al. 2000) and *Gli2*-homozygous *Gli3*-heterozygous mutant (Mo et al. 1997). In $Gli3^{Xtj}/Gli3^{Xtj}$ mice, more severe defects, three or more extra digits, were observed in 43% of the forelimb and 29% of the hindlimb. Furthermore, all the limbs of $Gli3^{Xtj}/Gli3^{Xtj}; Ski^-/+$ mice showed three or more extra digits. These results indicate that there is a genetic interaction between *Ski* and *Gli3*. We obtained only a very few $Gli3^{Xtj}/+; Ski^-/Ski^-$ embryos and no $Gli3^{Xtj}/Gli3^{Xtj}; Ski^-/Ski^-$ embryo at E11.5 (Supplementary Table 2), indicating that those types of mutant embryos die at an early stage.

Ectopic expression of Gli1 mRNA correlates with extra digits in $Gli3^{Xtj}/+;Ski^-/+$ mice

Because Shh blocks the Gli3 processing, the levels of $Gli3^{Rep}$ protein are higher in the anterior limb buds than in the posterior limb buds (Wang et al. 2000). Therefore, one possibility for the enhanced digit abnormalities of $Gli3^{Xtj}/+;Ski^-/+$ mice is that $Gli3^{Rep}$ represses a subset of target genes by interacting with Ski in the anterior region of limb buds. To examine this, we analyzed the expression of *Gli1* by in situ hybridization (Fig. 4A). In wild-type and $Ski^-/+$ forelimb buds, *Gli1* was expressed only in the posterior region, whereas in $Gli3^{Xtj}/+;Ski^-/+$ forelimb buds, it was also weakly expressed in the anterior region. The level of *Gli1* expression in the anterior region of the $Gli3^{Xtj}/+;Ski^-/+$ limb bud appeared to be higher than that in $Gli3^{Xtj}/+$ limb buds, but lower than that in $Gli3^{Xtj}/Gli3^{Xtj}$ and $Gli3^{Xtj}/Gli3^{Xtj}; Ski^-/+$ limb buds. To accurately measure the *Gli1* expression level, we prepared RNA from the anterior one-third and posterior one-third regions of limb buds, and quantitative reverse transcriptase–polymerase chain reacion (RT–PCR) was performed (Fig. 4B). *Gli1* expression levels in the anterior region of $Gli3^{Xtj}/+;Ski^-/+$ and $Gli3^{Xtj}/Gli3^{Xtj}$ limb buds were 115% and 125% higher than those of wild type, respectively, whereas there was no apparent difference in the *Gli1* mRNA level between $Gli3^{Xtj}/+$ and wild-type limb buds. Further, there was also no apparent difference in the anterior *Gli1* mRNA level between $Gli3^{Xtj}/Gli3^{Xtj}$ and $Gli3^{Xtj}/Gli3^{Xtj}; Ski^-/+$ (data not shown). Although Shh is ectopically expressed in the

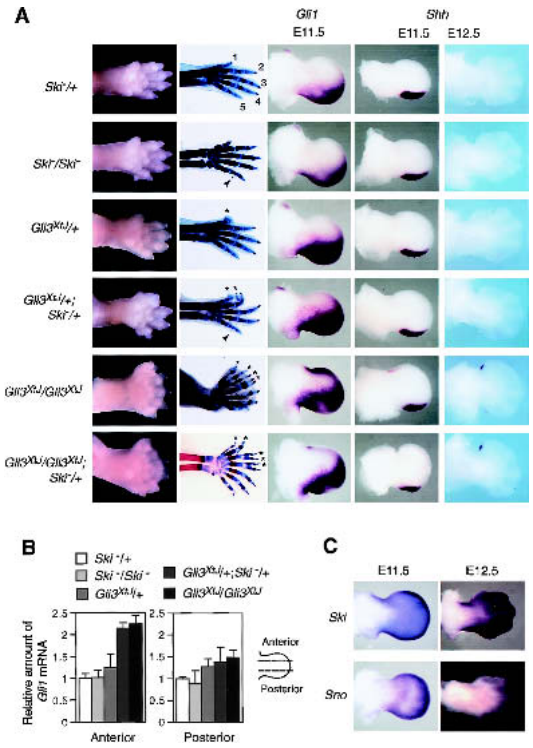

Figure 4. Genetic interaction between *Ski* and *Gli3*. (*A*) Skeletal phenotype of the forelimbs and expression of the *Gli1* and *Shh* in the forelimb bud. (*Left two* panels) Ventral and dorsal views of the forelimbs of E17.5 fetuses and newborn mice. Extra digits are shown by asterisks. A small posterior outgrowth is indicated by an arrowhead. Anterior is up. (*Right three* panels) Expression patterns of *Gli1* and *Shh* in E11.5 and E12.5 forelimb buds. Close-up view of whole-mount in situ hybridization of the right forelimb buds is indicated. (*B*) Level of *Gli1* mRNA. Total RNA was prepared from the anterior and posterior parts of the E11.5 limb buds, and *Gli1* mRNA was measured by the quantitative real-time PCR. The relative amount of *Gli1* mRNA compared with wild type is indicated by a bar graph. The level of *Gli1* mRNA in the posterior part was 10.6-fold higher than that in the anterior part. (*C*) Expression of *Ski* and *Sno* in the wild-type E11.5 and E12.5 limb buds. Close-up view of whole-mount in situ hybridization of the right forelimb buds is shown.

Table 1. *Relevant phenotypes of* Ski$^-$ *and* Gli3^{Xtj} *mutants*

Genotype	No. of pups	No. of anterior extra digits						Small posterior outgrowth	
		one		two		three or more			
Ski$^-$/*Ski*$^-$	7	0[a]	0%	0	0%	0	0%	13	93%
		0[b]	0%	0	0%	0	0%	4	29%
Gli3^{Xtj}/+	42	79[a]	94%	2	2%	0	0%	0	0%
		80[b]	95%	1	1%	0	0%	0	0%
Gli3^{Xtj}/+;*Ski*$^-$/+	32	16[a]	25%	48	75%	0	0%	37	58%
		54[b]	84%	7	11%	0	0%	6	9%
Gli3^{Xtj}/*Gli3*^{Xtj}	7	0[a]	0%	8	57%	6	43%	0	0%
		2[b]	14%	8	57%	4	29%	0	0%
Gli3^{Xtj}/*Gli3*^{Xtj};*Ski*$^-$/+	2	0[a]	0%	0	0%	4	100%	0	0%
		0[b]	0%	0	0%	4	100%	0	0%

[a]Forelimb; [b]hindlimb.

anterior region of *Gli3*^{XtJ}/*Gli3*^{XtJ} limb buds at E12.5, as reported (Masuya et al. 1995; Büscher et al. 1997), no ectopic expression of Shh was observed in *Gli3*^{XtJ}/+;*Ski*$^-$/+ limb buds. Expression of *Ski* in limb bud was high until E11.5, and then restricted to interdigital region at E12.5 (Fig. 4C). On the other hand, expression of *Sno* mRNA was high in the entire limb bud until E11.0 and restricted to the distal region. High expression of *Sno* mRNA was sustained in tips at E12.5 except for the anterior region.

Our results demonstrate that Ski acts in pattern formation as a corepressor of Gli3^{Rep}. Ski also negatively regulates FL-Gli3 activity. Gli2 has the repressor domain in its N-terminal region, like Gli3 (Sasaki et al. 1999), and Gli2 and Gli3 have the redundant functions in skeletal patterning (Mo et al. 1997). Like the case of Gli3, therefore, a repressor form of Gli2 might be generated by proteolytic processing and Ski may also act as its corepressor. In fact, we observed that Ski directly binds to Gli2 and is required for the Gli2N-dependent transcriptional repression. Higher frequency of three or more extra digits in *Gli3*^{Xtj}/*Gli3*^{Xtj}; *Ski*$^-$/+ limbs than in *Gli3*^{Xtj}/*Gli3*^{Xtj} limbs may be due to a decreased activity of the Gli2 repressor form. Consistent with this, *Gli2* and *Gli3* are expressed throughout the whole region of limb buds at E10.5–E11.5, whereas *Gli1* is expressed in the posterior part (Büscher and Rüther 1998). Because the Shh–Gli pathway plays an important role in development of not only the limb bud but also of other organs, at least some abnormal pattern formations observed in *Ski*-deficient embryos (Berk et al. 1997; Colmenares et al. 2002) could be explained by decreased Gli3^{Rep} activity and/or increased FL-Gli3 activity. Gli3- and probably Ci-dependent transcriptions are regulated by the coactivator CBP (CREB-binding protein; Akimaru et al. 1997; Dai et al. 1999) and the corepressor Ski. Because full-length Gli3 can bind to both CBP and Ski, there may exist a regulatory mechanism that determines the specificity of Gli3 binding to these factors, depending on the nature of the signal.

Materials and methods

The experiments were performed as described in the following paragraphs, and details are described in the Supplementary Material.

Yeast two-hybrid screening

The yeast two-hybrid screening was performed as described (Nomura et al. 1999) using the two reporters containing the LexA-binding sites or the Gli-binding site (Sasaki et al. 1997). The N-terminal 613 amino acids of GLI3 or the N-terminal 641 amino acids of Gli2 were used as bait.

In vitro binding assays and coimmunoprecipitation

In vitro binding assays were done using GST–Ski and GST–Gli3CT2 as described (Dai et al. 1999). For coimmunoprecipitation, a mixture of the plasmids to express Gli3, Gli1, Ski, HDAC1, or PKA was transfected into 293T cells. Forty hours after transfection, cells were lysed, and lysates were immunoprecipitated using appropriate antibodies. The immunocomplex was analyzed by Western blotting using appropriate antibodies. For coimmmunoprecipitation of endogenous proteins, the lysates were prepared from the 11.5-dpc mouse fetuses and immunoprecipitated using anti-Ski antibody, followed by Western blotting using anti-GLI3.

Mammalian two-hybrid assays, subcellular localization, and antibody injection assays

The mammalian two-hybrid assays were done using the Gli-binding sites containing luciferase reporter (Sasaki et al. 1997) and the plasmid encoding a Ski–Vp16 fusion. The subcellular localization study and antibody injection assays were done essentially as described (Nomura et al. 1999).

Luciferase reporter assays and analysis of Gli1 expression

The luciferase reporter assays using the luciferase reporter containing the Gal4 site or the *Gli1* promoter (pHR-luc) were done as described (Dai et al. 1999). *Gli1* gene expression in MNS-70 cells were also examined as described (Dai et al. 1999).

Analysis of embryos and quantitative real-time PCR

Analysis of cartilaginous tissues of newborn mice and whole-mount in situ hybridization was performed essentially as described (Tanaka et al. 1997). Quantitative real-time PCR-based measurements of RNA abundance were carried out using gene-specific double fluorescent probes and LightCycler (Roche).

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